Curie-point Pyrolysis Mass Spectrometry Applied to Characterization and Identification of Selected *Bacillus* Species

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The use of pyrolysis mass spectrometry in the characterization and identification of *Bacillus* species was studied. Fifty-three strains of four closely related groups, *Bacillus subtilis*, *B. pumilus*, *B. licheniformis* and '*B. amyloliquefaciens*', were used in a study of both sporulated and non-sporulated cultures. Pyrolysis was carried out using a Pyromass 8-80, a novel pyrolysis mass spectrometer specifically designed for fingerprinting complex samples. The pyrolysis data obtained were analysed using multivariate statistical techniques. All four groups could be differentiated using data from non-sporulated cultures but the data from sporulated cultures did not separate *B. subtilis* from '*B. amyloliquefaciens*' or *B. pumilus*. In contrast, *B. licheniformis* was more clearly differentiated from the other three species using these data. Culture maturity affected the mass spectra obtained from non-sporulated cultures.

INTRODUCTION

Rapid and accurate identification of bacteria, within minutes, rather than hours or even days, is a major objective of diagnostic microbiologists. It has been shown for *Bacillus* species that classical identification techniques can be improved upon using a commercially available miniaturized test system (Logan & Berkeley, 1981). However, a minimum of at least several hours is needed to carry out identification procedures based upon inoculation of an organism into diagnostic media, incubation and then reading and interpretation of test results. In contrast, approaches to identification based upon physical methods should produce results more rapidly and those based on direct examination of whole cells are likely to save most time. Of the methods allowing this, analytical pyrolysis is the most promising. Furthermore there is every reason to believe that it is a technique that will be universally applicable in bacterial identification (Wieten *et al.*, 1981).

Pyrolysis is the thermal degradation of matter in an inert atmosphere to produce volatile fragments characteristic of the original material. Thus it can be used to prepare non-volatile materials for subsequent examination by methods such as gas-liquid chromatography and mass spectrometry which require volatile samples.

Pyrolysis gas-liquid chromatography (Py-GC) has been used for the identification and differentiation of many types of bacteria (Reiner *et al.*, 1972; Oxborrow *et al.*, 1977; Stack *et al.*, 1978). However, problems such as column ageing, lack of similarity in the performance of new columns (Sekhon & Carmichael, 1973) and an inability to obtain inter-laboratory reproducibil-

Abbreviations: CVA, canonical variates analysis; PCA, principal co-ordinates analysis; SDA, stepwise discriminant analysis.

ity (Coupe *et al.*, 1970) have led to increasing interest in pyrolysis mass spectrometry (Py-MS), as an alternative method which avoids some of these difficulties.

Meuzelaar & Kistemaker (1973) built a Curie-point pyrolysis mass spectrometer specifically for fingerprinting complex samples such as bacteria. This was later developed to produce a fully automated instrument (Meuzelaar *et al.*, 1976) which has been successfully used for discriminating a wide variety of bacterial types (Meuzelaar *et al.*, 1976; Borst *et al.*, 1978; Boon *et al.*, 1980).

The aims of the work reported in this paper were to investigate the feasibility of using a new fingerprinting instrument, the Pyromass 8-80, for the rapid characterization and identification of bacteria. The Pyromass 8-80 is similar in concept to the instrument developed by Meuzelaar and co-workers but it is based on a magnetic mass analyser rather than a quadrupole.

The *Bacillus subtilis* spectrum was chosen for study as it represents a set of four closely related groups: *B. subtilis*, *B. pumilus*, *B. licheniformis* and '*B. amyloliquefaciens*'. These organisms share many common properties and possess few characteristics by which they can be separated (Gordon *et al.*, 1973), thus they provide an exacting test of the discriminatory abilities of the Pyromass 8-80. Both sporulated and non-sporulated cultures were included in the study so that the discrimination achieved using both morphological states could be compared.

METHODS

Organisms and growth conditions. Eight strains of each of four groups of *Bacillus*: *B. subtilis*, *B. pumilus*, *B. licheniformis* and '*B. amyloliquefaciens*' were used. In addition, duplicates of 21 strains were included, giving a total of 53 cultures. The strains used are listed in Table 1. They were held on nutrient agar (Oxoid) slants in the University of Bristol culture collection. Non-sporulated organisms were obtained by plating on nutrient agar for 16 h at 35 °C. Only two strains, *B. subtilis* B0093 and *B. licheniformis* B0242 showed signs of sporulation after 16 h incubation at 35 °C. These were re-plated and incubated for 10 h, so that only non-sporulated cultures were examined. Sporulated cultures were obtained by growth for 7 d at 30 °C on the same medium to which 5 mg $\text{MnSO}_4 \cdot \text{l}^{-1}$ had been added.

Sample preparation. Clean Ni-Fe wires with a Curie-point of 510 °C were mounted in clean glass sample holders as described by Meuzelaar *et al.* (1975). Bacterial growth was picked up from the plate surface using a disposable plastic loop (Nunc Intermed, Kamstrup, DK-4000, Roskilde, Denmark) and applied to the wire in a band about 2–4 mm wide, leaving the end 3 mm uncoated. The wire was then retracted into the reaction tube and secured with a plastic cap, so that, when placed in the mass spectrometer, the coated region was located in the centre of the pyrolysis zone. The tube was then fitted into the mass spectrometer probe ready for pyrolysis. Each of the 53 cultures was grown in duplicate and two samples prepared from each plate, giving four samples for each culture. Plate replicate samples were pyrolysed in succession but duplicate cultures were pyrolysed separately.

Pyrolysis mass spectrometry. This was carried out using a Pyromass 8-80 (VG Gas Analysis, Middlewich, Cheshire, UK) (Fig. 1). Pyrolysis is done by the Curie-point technique, power being supplied by a Fisher Labortechnik, 1.5 kW, 1.1 MHz high frequency generator. The reaction tube is inserted into the RF coil using the instrument probe.

On pyrolysis, the pyrolysate passes into a gold-coated expansion chamber and enters the ion source as a molecular beam via a ceramic tube. This inlet system is heated to a pre-set temperature, 150 °C. The source is an electron impact Nier type, surrounded by a liquid nitrogen cooled baffle to trap high molecular weight organic contaminants. An 8 cm 80° single focussing magnetic sector instrument is used for ion deflection.

The mass range is scanned exponentially from m/z (mass/charge ratio) 300 to m/z 12. Ions are detected by an electron multiplier coupled with a fast response X10 pre-amplifier. The pulses generated are counted by a Camac-based system employing an LSI 11/23 mini-computer and the raw mass intensity data are stored on a floppy diskette. Normalized data can be output as full or restricted range mass spectra by a line-printer and/or an X-Y plotter. The time taken from insertion of the sample to completion of the spectrum print out is 5–6 min, depending on the amount of data collected.

The operational parameters of the instrument can be altered from the computer keyboard. For analysis of bacterial cultures, the operational parameters of the instrument were: pyrolysis temperature, 510 °C for 2 s; hold time, 60 s; scan time, 1.3 s cycle⁻¹; number of scans, 35; electron energy, 16 eV.

Data processing. Pyrolysis data were recorded on eight floppy diskettes, one for each group of strains, with data from sporulated and non-sporulated cultures being held on separate disks. The pyrolysis data were first transferred to an IBM 3033 mainframe computer. Data from each group of strains were studied separately to determine intra-group relationships and later combined with that from other groups for inter-group analysis.

Table 1. List of strains used in this study

Identity	No. in study of O'Donnell <i>et al.</i> (1981) and origin	No. in Bristol University <i>Bacillus</i> culture collection
<i>B. subtilis</i>	1 DSM 10 (neotype)	B0014 B0225
	2 Gibson 636	B0092
	3 Gibson 1111	B0093 B0227
	4 Gibson 1156	B0095 B0228
	5 Gibson 1137	B0094 B0229
	6 Gibson 1115	B0230
	7 Gibson 1136	B0231
	8 Gibson 1152	B0232
<i>B. pumilus</i>	9 DSM 27 (type)	B0019 B0233
	10 Gibson 1130	B0103 B0234
	11 Gibson 1036	B0102 B0235
	12 Gibson 10	B0100 B0236
	13 Gibson 47	B0101 B0237
	14 Gibson 67	B0238
	15 Gibson 604	B0239
	16 Gibson 768	B0240
<i>B. licheniformis</i>	17 DSM 13 (neotype)	B0017 B0241
	18 Gibson 307	B0242
	19 Gibson 1142	B0243
	20 Gibson 1174	B0244
	21 Gibson 46	B0089 B0245
	22 Gibson 1160	B0091 B0246
	23 Gibson 5	B0088 B0247
	24 Gibson 1158	B0248
' <i>B. amyloliquefaciens</i> '	25 <i>B. megaterium</i> 203	B0169 B0249
	26 Fukumoto strain F	B0168 B0250
	27 <i>B. subtilis</i> H	B0172 B0251
	28 <i>B. subtilis</i> K	B0170 B0252
	29 <i>B. subtilis</i> N	B0171 B0253
	30 ATCC 23843	B0174 B0254
	31 ATCC 23845	B0176 B0255
	32 ATCC 23842	B0173 B0256

Fig. 1. Schematic representation of the Pyromass 8-80.

Fig. 2. Flow diagram showing the main steps in data processing. Raw data grouped by strain into four sets (S, P, L and A).

The flow diagram in Fig. 2 shows the basic steps used in the analysis of the data. Two statistical packages were used, HILDA (Highly Interactive Linear Discriminant Analysis) and GENSTAT. HILDA is an in house Fortran IV program containing procedures for: normalization; error detection; data reduction by calculation of characteristicity values (characteristicity of a feature is expressed by the ratio 'variance of the feature between samples/variance of the feature within samples', i.e. a measure of its discriminatory value) (Eshuis *et al.*, 1977); exploratory univariate displays; stepwise discriminant analysis (SDA); identification using classification functions. GENSTAT is a general statistical package (Nelder, 1979) and was used for PCA and CVA.

The first stage in reducing the raw data loaded into the IBM 3033 was to eliminate those m/z values too small or too large to contain any useful information. Initially, 179 masses were selected for further reduction of the data from non-sporulated cultures. The masses used were m/z 33–199, 208–211, 224, 225, 242, 256, 257, 265, 279 and 280. It was later found that some of the masses below m/z 33 could provide useful information and these were subsequently included and used for reduction of the data from sporulated cultures. The extra masses included were m/z 16, 17 and 26–32. The selected masses were first normalized, to remove the effect of sample size (Hill, 1966), by expressing the values of all other peaks as a percentage of the largest one. Two reduced data sets were then produced: one by selecting, for individual samples, 50 masses on the basis of their characteristicity values; the other by first averaging the data from plate replicate samples and then selecting 50 masses in the same way.

The reduced data from the individual groups was analysed using PCA (Gower, 1971) to assess reproducibility, to detect outliers or aberrant analyses and to display within group clustering tendencies. The combined reduced data sets were also analysed using PCA, but this is an exploratory analysis rather than one which will discriminate between groups, unless the variation is extreme, so the discriminatory techniques of CVA and SDA were also applied. MacFie *et al.* (1978) used CVA to measure distances between groups of strains using the generalized distance, Mahalanobis D^2 (Mahalanobis, 1936). Both CVA and SDA require an a priori group structure. That used was the arrangement based on API tests (O'Donnell *et al.*, 1980; Logan & Berkeley, 1981).

SDA successively selects masses to create a subset of masses that maximally discriminate the input groups. Classification functions are computed for each sample as are the D^2 values from each sample to the centre of each group. These show how well (or badly) a sample fits into its assigned group. SDA can also identify unknown strains by calculating classification functions using a subset of masses chosen by SDA from a data set of strains whose

identity is known. These functions allow the unknowns to be placed in the most probable group and the closeness of the unknown to this group can be determined from its D^2 value.

RESULTS

Reproducibility

It is, as yet, too early to assess the long term reproducibility of the Pyromass 8-80, but it was assessed over the term of this study by calculating the dissimilarity values for all plate replicate samples from the similarity coefficient, S_G (Gower, 1971). Figure 3 displays a histogram of the range of dissimilarity values for plate replicate samples from non-sporulated cultures. Dissimilarity was calculated as, $d = 100 - \%S$. The mean dissimilarity obtained (4%) compares favourably with that obtained by Windig *et al.* (1979) for the short term reproducibility (6%) and long term reproducibility (8%) of averaged pyrolysis mass spectra from glycogen and bovine serum albumin. It should be noted that although the mean dissimilarity is 4%, the distribution of values around the mean is asymmetrical and that a small number of highly dissimilar replicates cause the mean value to be larger than would be the case if the distribution were symmetrical.

Results from non-sporulated cultures

Typical spectra. Pyrolysis mass spectra of a representative strain of *B. pumilus* (B0101) and '*B. amyloliquefaciens*' (B0254) are shown in Fig. 4. Differences between the spectra can be observed by eye, for example at masses 95, 98, 122 and 125, but multivariate statistical techniques are essential for handling and comparing such information objectively.

Principal co-ordinates analysis. Some of the non-sporulated cultures had grown poorly after 16 h incubation and did not allow collection of two normal samples. In these instances smaller samples had to be used. PCA of the data from non-sporulated cultures showed that these small samples (giving < 110000 ions) tended to be outliers, that is, lay away from the main cluster of strains in their particular group. This effect was most noticeable in PCA on the individual group data (Fig. 5a) but was also seen in the PCA of the combined data (Fig. 5b), where inclusion of the outliers has caused the groups to be very diffuse. The analyses were repeated, omitting the data relating to small samples. Without these, the remaining points in the PCA plots for individual groups became more spread-out as they were not being compressed in order to fit

Fig. 3. Histogram of the range of dissimilarity values between plate replicate samples from non-sporulated cultures. The mean dissimilarity is 3.95%.

Fig. 4. (a) Pyrolysis mass spectrum from a non-sporulated sample of *B. pumilus*, B0101. (b) Pyrolysis mass spectrum from a non-sporulated sample of '*B. amyloliquefaciens*', B0254.

outliers onto the graph. This can be seen in Figs 5(a) and 6(a) which show, as an example, the change in the plots for the '*B. amyloliquefaciens*' group resulting from removal of outliers. Furthermore, the plots for each group were seen to split up into smaller clusters. The effects of removing the small samples were less noticeable, although still evident, in the PCA of the combined data (compare Fig. 5b with Fig. 6b), where the groups have become more defined, pulling in on themselves and away from each other. The effect is most noticeable in the *B. pumilus* group of strains.

Canonical variates analysis. Outliers also affected the discrimination of the groups by CVA. The effect of removing data relating to small samples from the analyses can be seen in the change in the CVA plots of combined data (see Figs 5c and 6c). After the removal of outliers, all four groups of strains could be discriminated, whereas before they overlapped; the

Fig. 5. (a) PCA plot of the '*B. amyloliquefaciens*' strains, using data from non-sporulated cultures. Broken lines join samples from duplicate plates and unbroken lines join samples from the same plate. (b) PCA plot of all four groups, using data from non-sporulated cultures. (c) CVA plot of all four groups, using data from non-sporulated cultures. For D values between the group means, see Table 2(a). See Table 2 legend for key to letters.

Fig. 6. (a) PCA plot of the '*B. amyloliquefaciens*' strains, using data from non-sporulated cultures after the removal of data from small samples. Broken lines join samples from duplicate plates and unbroken lines join samples from the same plate. (b) PCA plot of all four groups, using data from non-sporulated cultures after the removal of data from small samples. (c) CVA plot of all four groups, using data from non-sporulated cultures after the removal of data from small samples. For D values between the group means, see Table 2(b). See Table 2 legend for key to letters.

Table 2. *D* values calculated by CVA of data from non-sporulated and sporulated cultures

D values between given pairs of group means were calculated by CVA, using data from: (a) non-sporulated cultures before the removal of data from small samples; (b) non-sporulated cultures after the removal of data from small samples; (c) sporulated cultures. A *D* value of 5.8 *D* units, or over, indicates that the two populations can be discriminated with 95% confidence. (S represents *B. subtilis*; P represents *B. pumilus*; L represents *B. licheniformis*; A represents '*B. amyloliquefaciens*'.)

(a)					(b)					(c)				
S	0.0				S	0.0				S	0.0			
P	7.0	0.0			P	8.5	0.0			P	4.6	0.0		
L	6.0	6.9	0.0		L	7.6	7.3	0.0		L	11.5	12.8	0.0	
A	5.9	5.6	6.9	0.0	A	8.0	9.8	10.5	0.0	A	5.1	6.2	11.9	0.0
	S	P	L	A		S	P	L	A		S	P	L	A

Table 3. *D* values showing the effect on the discrimination of the groups of using different data sets for CVA

D values between given pairs of group means were calculated by CVA of different reduced data sets from non-sporulated cultures. The reduced data were produced by selecting 50 masses on the basis of characteristicity from a set of masses *m/z* 33–199, 208–211, 224, 225, 242, 256, 257, 265, 279 and 290, with the following changes: (a) masses 1–22 were included; (b) masses 43, 44 and 48 were omitted; (c) masses 16, 17 and 26–32 were included. See Table 2 legend for key to letters.

(a)					(b)					(c)				
S	0.0				S	0.0				S	0.0			
P	8.6	0.0			P	9.0	0.0			P	8.2	0.0		
L	8.2	6.2	0.0		L	8.9	7.1	0.0		L	9.0	8.4	0.0	
A	7.3	4.8	7.6	0.0	A	7.6	9.8	10.6	0.0	A	8.9	9.6	10.3	0.0
	S	P	L	A		S	P	L	A		S	P	L	A

'*B. amyloliquefaciens*' and *B. pumilus* groups not being distinguishable and the *B. subtilis* group being barely distinguishable from the *B. licheniformis* and '*B. amyloliquefaciens*' groups.

The Mahalanobis distance *D* between the centroids of any two groups must be above 5.8 *D* units for the two populations to be discriminated with 95% confidence. This figure was calculated using three canonical variates with two degrees of freedom, using the χ^2 procedure. Three canonical variates are required as we have four groups to discriminate. The group means however, are significantly discriminated at a distance of only 4.65 *D* units, but as we are interested in the discrimination of whole populations or species, the distance of 5.8 was used as our criterion for discrimination. From Fig. 6(c) it appears that the *B. subtilis* and *B. licheniformis* groups overlap, but it can be seen from Table 2 that the distance between their group centroids is 10.5 *D* units, so they are distinct groups. The CVA plot displays only the variation accounted for by the first two canonical variates. If the third canonical variate was included in the plot it would enable the separation between these two groups to be seen clearly.

The data were also analysed using a number of reduced data sets produced by including different combinations of masses in the data reduction exercise. In one analysis all of the lower masses were included (*m/z* 1–22); in another the three largest masses were removed (*m/z* 43, 44 and 58) and finally, only a few of the lower masses were included (*m/z* 16, 17, 26–32). These combinations of masses were used to compute reduced data sets from the averaged plate replicate data, which were then submitted to CVA. The resulting *D* values (Table 3) showed that including all the lower masses or removing the largest masses did not enhance the discrimination obtained using the original set of masses. However, including only a select few of the lower masses did slightly improve discrimination of the four groups of strains and it was for this reason that these selected masses were included when analysing the data from sporulated cultures.

Stepwise discriminant analysis. SDA of the data before the removal of outliers, did not (on the basis of *D*² values) place 30 of the 106 samples in the groups assigned to them in the a priori

Table 4. *Classification achieved by SDA of data from non-sporulated cultures*

Samples were allocated to their most probable group using classification functions calculated by SDA of data from non-sporulated cultures (a) before and (b) after the removal of data from small samples. See Table 2 legend for key to letters.

(a) Group	Strains put into group:				No. misplaced
	S	P	L	A	
S	18	2	3	1	6 from 24
P	4	14	2	6	12 from 26
L	2	2	20	0	4 from 24
A	2	4	2	24	8 from 32
Total	26	22	27	31	30 from 106

(b) Group	Strains put into group:				No. misplaced
	S	P	L	A	
S	16	1	1	0	2 from 18
P	0	19	0	1	1 from 20
L	0	0	22	0	0 from 22
A	1	0	0	25	1 from 26
Total	17	20	23	26	4 from 86

structure. It can be seen that the *B. pumilus* group of strains was the group in which most misplacing occurred (Table 4). This was also the group which contained most small samples. After the removal of these small samples only four of the remaining 96 samples were misplaced. A total of 16 masses were needed to achieve this level of accuracy in the discrimination of the four groups. The masses chosen by SDA were, in order of their discriminatory ability, m/z 39, 70, 126, 87, 171, 147, 83, 97, 61, 81, 88, 50, 34, 33, 111 and 43. Using the chosen 16 masses, data from eight non-sporulated cultures were analysed as if from 'unknown' strains and the samples assigned to their most probable groups, on the basis of their D^2 values. All eight strains were correctly identified.

Results from sporulated cultures

Principal co-ordinates analysis. In contrast with the findings from non-sporulated cultures, the small samples from sporulated cultures did not tend to be outliers. Analysis of the data from sporulated cultures showed that the PCA plots of the individual groups (Fig. 7a) and of the combined data (Fig. 7b) were similar to those obtained using data from non-sporulated cultures, after the removal of outliers.

Canonical variates analysis. CVA on the combined data from sporulated cultures (Fig. 7c) showed that the *B. licheniformis* group of strains was well discriminated from the other three groups, but that these were not, or were only just, discriminated. This contrasts with the results of CVA on non-sporulated cultures.

Stepwise discriminant analysis. SDA of the mean-reduced data from sporulated cultures reflected the fact that the *B. subtilis*, *B. pumilus* and '*B. amyloliquefaciens*' groups of strains overlapped (as seen in CVA of these data). The error involved in attempts to place strains in the groups of the a priori structure was 21%, that is, 21 of the 100 samples were misplaced.

DISCUSSION

If Py-MS is to be used routinely for the identification of micro-organisms, all aspects of reproducibility of the technique and the soundness of the data-handling procedures must be demonstrated. To allow data exchange between laboratories, inter-laboratory reproducibility

Fig. 7. (a) PCA plot of the '*B. amyloliquefaciens*' strains, using data from sporulated cultures. Broken lines join samples from duplicate plates and unbroken lines join samples from the same plate. (b) PCA plot of all four groups, using data from sporulated cultures. (c) CVA plot of all four groups, using data from sporulated cultures. For D values between the group means, see Table 2(c). See Table 2 legend for key to letters.

must be of a high order. The Pyromass 8-80 has been shown to behave reproducibly over the short-term of this study, with a mean dissimilarity of 4% for pyrolysis mass spectra from replicate samples. The instrument has only recently been put into operation and is the only one of its type, so the long-term and inter-laboratory reproducibility cannot yet be determined.

Statistical data-handling procedures, involving multivariate analyses are an essential part of the technique, as otherwise the large amounts of complex data generated cannot be analysed adequately. The removal of outliers from a data-set is an established mathematical practice, but there is also a microbiological justification for doing this. It has been shown by other workers that the quantity of pyrolysis products formed from secondary reactions is directly related to the quantity of material pyrolysed (Jones & Moyle, 1961; Simon & Giacobbo, 1965), but that spectra can be compared directly by using normalization procedures to remove the effect of sample size (Hill, 1966). It was therefore surprising that small and large samples from non-sporulated cultures of the same strain produced spectra that were markedly different. As this was not found to be the case with samples from sporulated cultures, variance could not be due to differences in sample size, but rather must be a reflection of some real difference between the samples.

Small samples were taken only from cultures that had not produced a large amount of growth, making it difficult to collect two large samples. Poorly-grown non-sporulated cultures resulted either from having a longer lag time in the germination of spores than that of organisms on well-grown plates or because the strains were slow-growing. Thus the difference seen between small and large samples was due to the samples being taken from cultures at different stages of maturity. All the samples from sporulated cultures came from cultures which had been incubated for 7 d and had reached the sporulation stage, so that there were no differences in the maturity of large and small samples.

When the small samples were removed from the data relating to non-sporulated cultures, the resulting improvement in the discrimination of the four groups of strains confirmed that these aberrant results had been confusing the intrinsic group structure. It seems then, that it is not only necessary to standardize the culture conditions and sample preparation for Py-MS, but also to standardize the growth phase of cultures used. Such standardization is likely to be more of a problem with sporulating cultures of organisms such as *Bacillus* and *Clostridium*. Thus, as discrimination of the four groups was better using non-sporulated than sporulated cultures, non-sporulated cultures should be used for analysing *Bacillus* species. This also has the advantage of allowing identification at an earlier stage.

The results of this study show that the four groups of strains can be discriminated by Py-MS if non-sporulated cultures are used. This is in accord with the results of the Py-GC study conducted by O'Donnell *et al.* (1980). '*Bacillus amyloliquefaciens*' is currently not regarded as a species separate from *B. subtilis*, though O'Donnell *et al.* (1980) and Welker & Campbell (1967*a, b*) have been able to distinguish them clearly and have suggested that it should be. Other workers (Gordon *et al.*, 1973; Smith *et al.*, 1952) however, have found these organisms to be indistinguishable. Our results thus strengthen the case for the separation of '*B. amyloliquefaciens*' from *B. subtilis*. When sporulated cultures were analysed using Py-MS, only *B. licheniformis* could be completely differentiated. It was expected that samples from sporulated cultures would prove to be more similar, as sporulating organisms are in a resting state with many of the genes that would normally code for characteristics that can be used to separate the organisms unexpressed. It is interesting therefore that *B. licheniformis* is more clearly separated from the others using sporulated cultures. Why this is so is unclear.

The sub-clusters of strains shown in the PCA plots of the individual groups may represent pyrotypes of each species, that is, sub-sets of strains that can be discriminated reproducibly by Py-MS. Further studies need to be carried out before this can be reliably ascertained.

Although the data base assembled for the non-sporulated cultures contained relatively few pyrolysis mass spectra, it was possible, using SDA, to place all but four of the strains into their respective groups. Moreover, eight strains treated as unknowns were correctly identified. Although it will take some time to compile the necessary data base and to test the long term stability of the Pyromass 8-80, rapid, accurate identification of *Bacillus* species from grown colonies is expected to be achievable in the near future.

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